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Leukocyte response and phagocytic activity in Nile tilapia experimentally infected with *Enterococcus* sp.

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Abstract This study evaluated the total and differential leukocyte counting and the phagocytic activity in Nile tilapia *Oreochromis niloticus* experimentally injected with *Enterococcus* sp. in the swim bladder. Fish were distributed in four treatments in triplicates of non-injected fish, fish injected with 1 ml of sterile saline solution 0.65%, and fish injected with 1×10^3 and 1×10^6 colony-forming units (CFU) of *Enterococcus* diluted in 1 ml sterile saline. Twenty-four hours after injection, the fish were anesthetized and the blood collected for white blood cell (WBC) counts, differential counting of WBC, and phagocytic activity of blood leukocytes. The increased numbers of WBC and lymphocytes were followed by decreased number of monocyte after infection. The percentages of phagocytic activities in the blood were 55.3 and 55.9%, respectively, in tilapia injected with 1×10^3 and 1×10^6 CFU/ml.

Keywords Infection · Leukocyte · Phagocytosis · Tilapia

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Introduction

With the rapid increase in fish production important problems concerning fish health are beginning to appear . Some of these are clinical symptoms related to altered hematological parameters (Moraes and Martins 2004). Tilapia is one of the most reared freshwater fish throughout the world (Cavichiolo et al. 2002). Beside parasitic diseases, bacterial infections have compromised the success of fish production. Hematological parameters are among one of the important tools for fish disease diagnosis (Ruane et al. 2000; Ranzani-Paiva et al. 2005; Ghiraldelli et al. 2006). For example, Tavares-Dias et al. (2002) observed increases in the neutrophill and monocyte numbers followed by decreased lymphocyte numbers in diseased tilapia. Studies on the hematological responses in the salmonid *Oncorhynchus keta* (Haney et al. 1992) and the cichlid *Etroplus suratensis* (Pathiratne and Rajapakshe 1998) in fish respectively artificially infected with erythrocytic necrosis virus and epizootic ulcerative syndrome,

registered an increase in white blood cell (WBC) numbers. In carp, experimentally infected with bacteria, the WBC count was also significantly increased 7 days after infection according to the results of Siwicki and Studnicka (1987).

Phagocytosis is a defense reaction of the organisms that may vary according to fish health status (Toranzo et al. 1995). These authors pointed out increased phagocytic activity in the spleen of turbot *Scophthalmus maximus* injected with bacterin. Observations of Cai et al. (2004) revealed higher responses of phagocytic activity in the blood of Nile tilapia *Oreochromis niloticus* (61%) than that observed in blue tilapia *O. aureus* (39%) after infection by *Aeromonas sobria*.

However, in Brazil, little is known on the effects of bacterial infection in reared tilapia and its consequences on the hematological parameters and phagocytosis. According to Anderson (1990), phagocytic activity is considered a good nonspecific immunological indicator to determine the immunosuppression of fish. In order to verify the response of Nile tilapia to experimental infection with *Enterococcus* sp., this assay was performed to determine whether there exists an influence on the WBC count and phagocytic activity of blood leukocytes.

Material and methods

Sixty Nile tilapia (269.0 ± 63.6 g weight and 23.4 ± 2.0 cm length) were distributed into four groups with 15 fish in each group, maintained in 12 tanks (150-l capacity) each containing 5 fish (each group in triplicate). Before assay, fish were acclimatized for 10 days and fed with commercial diet. During this period, the water quality was maintained at temperature 22.0 ± 0.2°C, pH 7.0 ± 0.2, ammonia 0.5 ± 0.1 mg/l, and dissolved oxygen 4.1 ± 0.5 mg/l. The bacterium was originally isolated from diseased tilapia raised in cages in the São Francisco River, AL, and the inoculums prepared according to Martins et al. (2008).

The groups were: non-injected fish; fish injected with 1 ml of 0.65% sterile saline solution; and fish injected with 1×10^3 and 1×10^6 CFU of live *Enterococcus* diluted in 1 ml saline solution in the swim bladder, according to Matushima and Mariano (1996) and Martins et al. (2004).

Twenty-four hours after injection the fish were anesthetized with benzocaine solution (50 mg/l) and blood was withdrawn from the caudal vessel into a syringe containing a drop of 10% EDTA solution (approved by Ethic Committee no. 23080.007045/2006-61/UFSC) for total count of WBCs by the indirect method (Martins et al. 2004), differential counting of leucocytes in the smears stained by Giemsa/May-Grunwald (Rosenfeld 1947), and phagocytic activity of circulating blood. Total leukocytes number was calculated by the formula:

Leukocytes/μl = (leucocyte number in the smear × erythrocyte number/μl)/2,000 erythrocytes counted in the blood smear.

Leukocyte phagocytic function followed the method of Cai et al. (2004) slightly modified as follows: after blood collection, 0.5 ml of blood was dropped into centrifuge tubes, to which was added 0.25 ml of 1×10^6 *Enterococcus* suspension before shaking. The tubes were kept at 28°C in a water bath for 30 min, and shaken every 10 min. After this time, in order to centrifuge as recommended by Cai et al. (2004), the blood smears were done in duplicates just after incubation and stained by Giemsa/May-Grunwald (Rosenfeld 1947). The number of leukocytes that engulfed bacteria was counted as percentages in relation to total leukocyte number in the smear from the phagocytosis assay. Statistical analysis was carried out considering each tank as the experimental unit. The values of differential counting in percentage are transformed in arc sin(x) before analysis. The results were submitted to the variance analysis (ANOVA) and F-test ($P < 0.05$), and the averages to the Tukey test ($P < 0.05$).

Results and discussion

This study found that fish injected with 1×10^6 *Enterococcus* showed increased numbers ($P < 0.05$) of lymphocytes, contrary to that observed in non-injected fish. In the circulating blood, the lymphocyte number of fish injected with 1×10^6 *Enterococcus* showed the highest values ($P < 0.05$) when compared to non-injected animals and to those injected with saline and 1×10^3 *Enterococcus* (Table 1). This result may be related to lymphocyte production in bacteria-injected fish to combat the infection.

Table 1 Mean values of WBCs, differential counting of leucocytes (in microliter and percentage) and phagocytosis percentage in Nile tilapia *Oreochromis niloticus* non-injected (*NI*), injected with saline, or with 1×10^3 or 1×10^6 CFU *Enterococcus*/ml in the swim bladder

Treatments	WBC ($\times 10^3$/μl)	Lymphocytes		Neutrophils		Monocytes		Phagocytosis (%)
		($\times 10^3$/μl)	(%)	($\times 10^3$/μl)	(%)	($\times 10^3$/μl)	(%)	
NI	15.0 ± 4.5b	26.5 ± 5.6a	56.9 ± 9.4a	6.5 ± 2.7a	13.5 ± 4.7a	14.3 ± 4.4ab	30.3 ± 5.3a	0
Saline	20.8 ± 12.0ab	28.1 ± 7.2a	51.6 ± 5.0a	14.7 ± 5.3b	25.3 ± 6.4a	12.5 ± 2.3ab	23.5 ± 2.9a	0
1×10^3	19.0 ± 11.0ab	34.0 ± 7.9a	61.1 ± 0.6a	5.6 ± 3.0a	10.1 ± 3.8a	17.3 ± 6.1a	28.7 ± 4.0a	55.3 ± 9.6a
1×10^6	27.3 ± 15.8a	48.1 ± 9.9b	78.3 ± 2.5a	2.9 ± 1.3a	4.9 ± 0.5a	9.9 ± 2.1b	16.8 ± 2.1a	55.9 ± 10.2a

Letters in the columns indicate significant difference among treatments ($P < 0.05$)

This study found a high ($P < 0.05$) percentage of phagocytosed bacterium by leucocytes in injected fish (Table 1). In the present assay, the phagocytic activity in the blood of tilapia injected with *Enterococcus* was similar to reported by Cai et al. (2004) in Nile tilapia. These results confirmed the fact that fish artificially stimulated by bacterial injection have their immune system activated. Furthermore, in contrast to lymphopenia observed by Balfry et al. (1997) and Lamas et al. (1994) in tilapia challenged with *Vibrio parahaemolyticus* and rainbow trout challenged with *Vibrio anguillarum*, respectively, the number of circulating lymphocytes increased in 1×10^6 injected fish. However, Balfry et al. (1997) and Lamas et al. (1994) used intraperitoneal injection. According to Lamas et al. (1994) lymphopenia may be related to migration of lymphocytes to tissues. In fact, fish injected with the highest doses of *Enterococcus* showed more lymphocytes in the circulating blood than the other fish groups.

Interestingly, fish injected with saline showed a higher number of neutrophils than the other treatments. On the other hand, the numbers of monocyte in fish injected with the bacterium are not different from saline-injected and non-injected fish (Table 1). Ranzani-Paiva et al. (2004) did not find changes in the neuthrophil and monocyte counts after experimental infection with *Mycobacterium marinum*. On the other hand, Garcia et al. (2007) observed an increase in the number of neutrophils and monocytes in *Piaractus mesopotamicus* after infection with *Aeromonas hydrophila*.

Pathiratne and Rajapakshe (1998) in cichlid fish severely infected by epizootic ulcerative syndrome and Harikrishnan et al. (2003) in carp infected by *A. hydrophila* observed increases in the leukocyte number. This is contrary to what has been observed in pacu *P. mesopotamicus* infected by *A. hydrophila* (Garcia et al. 2007) and in rainbow trout *Oncorhynchus mykiss* infected by *V. anguillarum* (Lamas et al. 1994). In this study, the number of WBC after injection with 1×10^6 *Enterococcus* was higher than in control fish. Nevertheless, it was similar to fish injected with 1×10^3 *Enterococcus* and saline (Table 1). This result suggests that an increase in number of these cells may be related to stress of handling and not due to bacterial injection. The number of WBC of tilapia non-injected, injected with saline, and with bacterium remained at the lower limit reported by Hrubec et al. (2000) in cultured tilapia.

This assay showed that immune system may be stimulated by, for example, inactivated bacteria. Future studies are necessary on Brazilian fish vaccination.

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